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       Fragments and Peptides to Breast Carcinomas

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The focus of this research is to generate and characterize novel human antibody fragments (Fabs) and peptides that bind breast cancer cells. A combinatorial approach is being taken to generate immunoglobulin Fab that recognize a specific determinant on breast cancer cells including the breast cancer-associated Thomsen-Friedenreich (T) glycoantigen. In addition, human Fab and peptides will be generated from combinatorial libraries that bind breast cancer cells, irrespective of defined cell surface markers. The hypothesis that is implicit in these studies is that combinatorial approaches will provide Fab and peptides specific to either defined or ill-defined antigens present on breast cancer cells. The goals of this study are to: 1) generate human IgG Fab that bind specifically to the T antigen expressed on breast cancers from pre-existing Fab, 2) isolate Fab that bind ductal and lobular breast carcinoma cell lines from human combinatorial phage Fab display libraries, and 3) isolate peptides that bind breast carcinoma cell lines from combinatorial phage peptide display libraries. Results suggest phage libraries can yield Fab and scFv that bind to T antigen. Peptides and Fab that recognize lobular and ductal breast cancer cells are presently being sought .

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FOREWORD

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Combinatorial Approach to the Isolation of Human Antibody Fragments and Peptides to Breast Carcinomas

Year One Annual Report

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(5) INTRODUCTION

Continued basic research in breast cancer is of fundamental importance since breast cancer is the leading cause of death in women (1). Breast cancer is known to develop from normal epithelium through several stages. It is not certain exactly when malignancy begins, however "invasive" breast carcinoma with metastatic potential occurs when epithelial cells invade the surrounding stroma (2). Invasive breast carcinoma may be preceded by noninvasive ductal or lobular hyperplasia or carcinoma. Preinvasive lesions as well as invasive breast tumors can often be detected by Mammography. Mammography has not been as reliable for the detection of these lesions in women with dense breast tissue, found commonly in pre-menopausal women. Therefore, a significant percentage of women may go undiagnosed by mammography. Alternative detection, diagnostic, and therapy regimens would be facilitated by reagents that can easily and sensitively detect breast cancer cell markers. The hypothesis that is implicit in these studies is that combinatorial human IgG and peptide libraries will provide molecules specific to antigens present on breast cancer cells. The Fab and peptides obtained will be valuable tools that will expedite basic and applied research into defining the determinants of breast cancer that may also facilitate early detection and diagnosis. The peptides and Fab that bind breast cancer cells, once radiolabeled, may be developed into important new therapeutic or imaging reagents. Novel approaches will be employed to generate new and improved human Fab that specifically recognize breast cancer cells. Fab immunoglobulin that recognize the well-documented breast cancer-associated Thomsen-Friedenreich (T) glycoantigen will be obtained. In addition, a combinatorial approach will be taken to isolate human Fab against breast cancer cells, regardless of detailed knowledge of target antigens. Combinatorial methods will also be applied for the isolation of small peptides that bind breast cancer cells. Both peptides and Fab that bind breast cancer cells are sought because it is difficult to predict which type of molecule will possess the most ideal properties for a diagnostic or therapeutic.

(6) BODY

The relevance of our proposed research is reflected in our goals of obtaining Fab and peptides that specifically and tightly bind breast cancer cells or cancer antigens. A limited number of murine monoclonal antibodies have been generated by others to the few known breast cancer-associated antigens, although none of them are cell and carcinoma specific. Moreover, the well documented immunogenicity and clearance problems of murine antibodies necessitates the development of 1) specific human Fab for application to human breast cancer and/or 2) the development of small molecule breast cancer-targeting agents. Our progress in the first year is summarized below.

Technical Objective 1: Generate human IgG Fab that bind specifically to the T antigen.

Task 1: Months 1-6: Generation of T Antigen Specific Fab by CDR Random Mutagenesis. Sequence Fab, and generate a database of T-binding sequences.
Research Accomplished: T antigen is exposed on many primary carcinomas (breast and colon) and their metastases, and the immunodominant portion is the terminal Galβ1-3GalNAcα carbohydrate moiety. T antigen is masked and inaccessible to the immune system on the surfaces of healthy cells, although it is exposed and immunoreactive on human carcinoma and T cell lymphoma cells. Peptides that bind the carcinoma associated T antigen have been previously isolated in our laboratory from a random 15 amino acid phage display library (3,4). It was our goal in this study to obtain Fab that bind T antigen as well. Immunoglobulin cDNA libraries from cancer patients with randomized HCDRs contained in the bacteriophage Fab display system pComb3 or single chain Fv (scFv) libraries from naive human immune repertoires (provided by Dr. Greg Winter, Cambridge, England) were affinity selected against T antigen to obtain IgG of human origin. Combinatorial IgG (k,λ) Fab libraries were constructed in the phage display vector pComb3, using protocols developed in our laboratory (5). Briefly, lymphocytes were obtained from patients undergoing leukophoresis and cDNA, synthesized from patient lymphocyte RNA, was used as template for PCR amplification of light chain and Fd (VH + CH1) DNA. PCR primer synthesis was based on a compilation of oligonucleotide primers used for PCR of human immunoglobulin genes.

Human IgG random CDR combinatorial Fab phage display libraries were screened for Fab that bound T antigen presented as a BSA-T antigen conjugant (15-20 moles of T/mole of BSA) or asialofetuin. Asialofetuin contains three natural O-linked T antigen structures per molecule. Initially, affinity selection

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was performed under non-stringent conditions (i.e. high antigen concentration). Since a library may only have one copy of a desired clone, it is strategically beneficial to affinity select under non-stringent conditions in the first round to insure it is not lost from the library. As the phage population is enriched for clones that possess the desired binding properties, more stringent selection conditions are employed (i.e. low antigen concentration, high salt, detergent, etc). Table 1 below lists the percent yields for each round of selection. After an initial decline in percent yield during the first three rounds of typical selection, there appeared to be an enrichment of antigen binding clones in the fourth round.

Table 1: Enrichment in Affinity Selection of Random HCDR Fab libraries Against T Antigen

Selection Round:	1	2	3	4
Percent Yield	7.5×10^{-3}	4.5×10^{-5}	3.3×10^{-6}	1.3×10^{-4}

After four rounds of affinity selection, individual phage clones were plated out and analyzed for antigen binding activity by filter binding assays. Soluble Fab were produced from clones exhibiting the strongest signal in the filter binding assays. The antigen binding affinities and specificities of the soluble Fab clones were determined by enzyme-linked immunosorbant assay (ELISA). In the ELISAs, Fab were coated on the bottom of microtiter wells at various concentrations. The microtiter wells were blocked with BSA and incubated with various concentrations of biotinylated asialofetuin. After incubation, the wells were washed extensively with phosphate buffered saline and detergent. The wells were incubated with a streptavidin-alkaline phosphatase conjugate and washed extensively. The assays were developed with the chromogenic substrate p-nitrophenyl phosphate. Optical densities of the wells were measured by absorption at 405 nm. Results from the ELISAs demonstrated that several of the Fab exhibited specific and concentration dependent binding to asialofetuin.

Six Fab DNA clones (9, 17, 22, 52, 53, 54) with the highest ratio of asialofetuin to BSA binding properties as determined by ELISA were analyzed also by micropanning to confirm their binding activities. Theoretically micropanning is more sensitive than ELISA. In micropanning one compares the phage outputs of clones bound to AF and to BSA (as control). Table 2 summarizes the results of micropanning.

Table 2: Micropanning of Selected Fab Clones

Clone #	output AF/output BSA at input 10^8	output AF/output BSA at input 10^9
52	2	6.18
53	10	4.25
54	3.48	3.05
9	5.3	3.06
17	7.75	2.78
22	2.8	3.29

The micropanning demonstrated that all selected clones had a higher affinity to AF than to BSA, but precise values have not yet been determined.

The six Fab DNA clones, (9, 17, 22, 52, 53, 54) with the highest ratio of AF binding to BSA were next DNA sequenced. Analysis of the H chain sequence revealed that 5 out of the 6 clones had the same H chain DNA sequence. Clone 52 had a different heavy chain sequence but its CDR3 was homologous to the other clones. The L chains of the Fab were all different from each other, however.

Phage #	Sequence
9,17,22,53,54	Val-**Tyr-Cys-Ala-Lys-Met**-Val
52	Tyr-**Tyr-Cys-Ala-Lys-Met**-Arg

These results suggest a common antigen recognition motif. The isolation of 5 Fab with identical heavy chain

sequences but different light chains indicates successful enrichment of Fab during the affinity selection process. Several more clones will be sequenced to confirm this pattern.

The naive human scFv phage display library was also screened for antibody fragments that bound the carcinoma-associated T antigen. The human scFv library has a diversity of 1×10^{10} and several antibody fragments with nanomolar affinities have been isolated from it (6). Phage display libraries with higher diversities usually yield antibody fragments with greater affinities than libraries with lower diversities. Strategies for screening the scFv were similar to those described for screening the Fab. The scFv library was screened with 4 rounds of affinity selection alternating between polyacrylamide-T and HSA-T glycoconjugates. Table 3 illustrates the significant increase in percent yield of the libraries during the rounds of affinity selection indicating that there was enrichment for scFv that bound the glycoantigens.

Table 3: Enrichment of scFv Library Affinity Selected Against T Antigen

Selection Round:	1	2	3	4
Percent Yield				
-T antigen	1.5×10^{-4}	6.4×10^{-4}	2.9×10^{-3}	5.3×10^{-1}

Eluant from the last round of T antigen affinity selection was plated out and individual scFv clones were isolated. The phage clones were screened for antigen binding by ELISA. The ELISAs were performed as previously described except that phage were coated on the bottoms of microtiter wells. The titration ELISA for one of the scFv clones is shown in Figure 1.

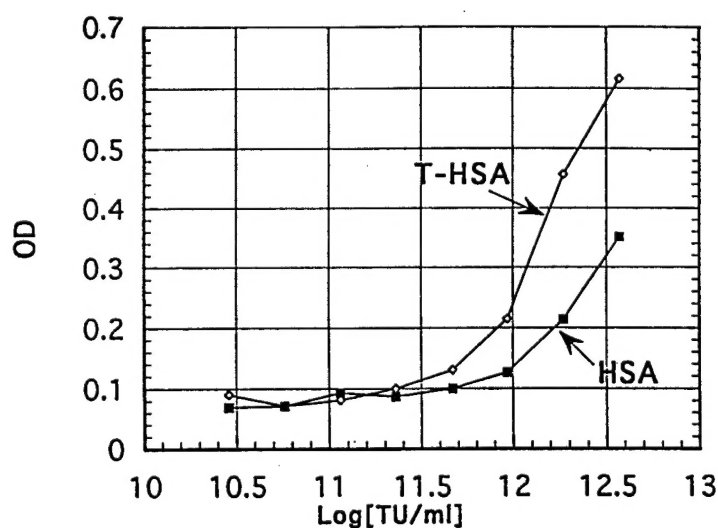Figure 1: Binding of scFv Phage Clone to T Antigen

Figure 1. Binding of phage expressing scFv to T antigen. An ELISA was performed with immobilized HSA as control (filled in square) or HSA-T conjugate (open square). A phage clone expressing an scFV affinity selected against T antigen was added. Binding was measured colorimetrically.

Task 2: Months 6-9: Isolate anti-T IgM from murine hybridomas and isolate human IgM from human sera or phage display libraries.
Research Accomplished: It was originally proposed to isolate IgM hybridomas obtained from collaborator George Springer. Unfortunately, he died recently and all cell lines and hybridomas were bequeathed to a senior investigator who will no longer make them available. As an alternative, we have initiated the production of anti-T hybridomas in mice through injection of T-BSA and cancer cell lines expressing T antigen on their surfaces. It is too early in the procedure to determine if high affinity anti-T specific antibodies will be obtained.

Task 3: Months 9-12: Evolution of a T Antigen-Specific Fab by DNA shuffling. Sequence Fab, express Fab in *E. coli*.

Research Accomplished: We have begun shuffling of a non-specific Fab that binds T antigen. All CDR domains were shuffled, and chimeric Fab were purified using anti-L chain affinity chromatography (7). Nitrocellulose filter binding experiments are underway. The chimeras have varying relative affinities for T antigen. It is unclear yet how specific they are for the carbohydrate portion of T. Based on our work with DNA shuffling with Fab, and the work of Stemmer and co-workers (8), it may be difficult to convert the non-specific Fab into one that is completely specific for T. It is our belief that strategies outlined in Tasks 1 and 2 of this objective or Tasks 1 and 2 of objective 2 may yield more promising antibodies.

Task 4: Months 12-18: Purify Fab using affinity chromatography and examine binding to T antigen by ELISA, immunofluorescence and fluorescence titration.

Research Accomplished: We are ahead of schedule with this task in that we have expressed several Fab and scFv in *E. coli*, and purified several. Expression of four anti-T selected soluble scFv was achieved in *E. coli*. The scFv were secreted into both the media and the periplasm. Soluble scFv were produced from phage clones exhibiting the best ELISA results. The scFv contain a His-tag so they were readily purified from *E. coli* using nickel affinity chromatography. Purified scFv molecules were analyzed for their binding affinities and specificities by titration ELISAs. The assays yield a relative binding constant of the scFv and demonstrate specificity of antigen recognition.

Technical Objective 2: Isolate Fab that bind ductal and lobular breast carcinoma cell lines from human combinatorial Fab and peptide phage display libraries.

Task 1: Months 1-2: Obtain cell lines, grow cells, make frozen stocks and extracts.
Research Accomplished: MDA-MB 435, MDA-MB-468 - lobular breast cancer cell lines, Hs578T a ductal cancer cell line, and Hs578Bstneg a normal ductal cell line were purchased. MDA-MB 435 and MDA-MB 468 were routinely grown in RPMI-1640 media adjusted to 4 mM L-glutamine and fetal bovine serum, 10% v/v. Hs578Bst cells were routinely grown in a supplemented media obtained from ATCC called Hybricare. To this media was also added epidermal growth factor at a concentration of 30 ng/ml, and fetal bovine serum, 10% v/v. Hs578T cells were grown in Dulbecco's modified Eagle's medium adjusted to contain 4 mM L-glutamine, 1.0 mM sodium pyruvate, bovine insulin at a concentration of 10 ug/ml and fetal bovine serum, 10% v/v.

Cells were passed at least once weekly (or when necessary) when cells had reached approximately 80% confluency, using standard protocols. Frozen cell stocks were also prepared using media supplemented with dimethyl sulfoxide (5% v/v) and cells were stored at -80 C°.

Task 2: Months 3-12: Affinity select phage Fab libraries against cell lines.
Research Accomplished: Each of the cell lines to be screened was grown in small 35 mm diameter tissue culture dishes under normal growth conditions until at least 80% confluency was obtained. Preliminary experiments showed that if the growth media was subsequently removed and replaced with either tris buffered saline (TBS) or TBS supplemented with 0.05% tween-20 (TBST) then over the course of a 2-3 hour incubation at room temperature, many of the cells would detach from the plates. Each of the cell lines behaved slightly differently in this respect, with some being less resistant to dislodgement than others.

This observation led us to believe that if phage were added in TBS to the cells, then over the course of subsequent incubations and washing stages, many of the cells and thus possible phage binding these cells would be lost. Thus the following alternative approach was used.

Phage (approximately 10^{10} virions) from a phage Fab display library displaying Fab on its pIII coat protein, were added to each dish of cells containing growth media. It was thought that by allowing the phage to also interact with the media which would subsequently be removed we might be able to remove some of the phage particles that recognize serum components such as albumin that are present in relatively large amounts.

After 2-3 hrs incubation at room temperature with gentle rocking, the media was used to dislodge cells that remained bound to the dishes. This was achieved simply by pipetting media around the dish. The media containing cells were then transferred to 1.8 ml microcentrifuge tubes. Each tube was centrifuged at 1500 x g for 2-4 minutes to obtain a small cell pellet. The cell pellet was washed by the addition of TBS, and the pellet was resuspended by gentle pipetting. The steps of centrifugation and resuspension were repeated several

times. In the first round of biopanning, it is essential that we try to obtain all the phage that have an affinity for the target (in this case the cells). This is due to the fact that each of the various phage particles is only represented by perhaps 1-10000 copies. Thus, the length of incubation is increased and the number and stringency of the wash stages is minimized.

Following the wash stages, specifically bound phage were eluted by the addition of glycine-HCl pH 2.2 with resuspension and gentle agitation for approximately ten minutes. The suspension was then briefly centrifuged and the supernatant carefully removed. The supernatant was neutralized with the addition of 1 M Tris-Cl pH 12. Titering of the isolated phage was carried out essentially according to established protocols. A small portion of the eluted phage was used to produce serial dilutions of the phage which were subsequently used to infect XL-1 Blue *E. coli* cells. Cells were then plated on agar plates containing kanamycin and tetracycline - XL-1 Blue cells carry resistance to kanamycin and the phage that infect the cells will confer tetracycline resistance to those cells. Following overnight incubation the colonies were counted and a percentage yield of phage isolated to those input may be calculated. The remaining portion of the first round eluted phage was used to infect *E. coli* cells which were subsequently used to produce phage particles to be used as the input phage for biopanning round #2. Phage were produced using standard procedures, removal of bacterial cells by low speed centrifugation and precipitation of phage using polyethylene glycol followed by high speed centrifugation. A portion of these amplified phage were used for titering to obtain a starting value for the next round.

Preliminary results would suggest that this approach works so far as that phage are obtained at the end of each round of biopanning. We have completed two rounds of affinity selection and will perform two more round before analyzing the clones.

Task 3: Months 13-16: Identify clones that bind cell lines using colony hybridization.
Will begin this in the next month.

Task 4: Months 17-24: Sequence Fab genes, express Fab and purify using affinity chromatography, and characterize binding using immunofluorescence and fluorescence titration. We should be on schedule for this.

Technical Objective 3: Isolate peptides that bind breast carcinoma cell lines from combinatorial phage peptide display libraries.

Task 1: Months 25-28: Affinity select phage peptide libraries against cell lines.
Research Accomplished: Since we have not yet completed Task 2 of objective #1 and are waiting for mice to illicit immune response, we decided to begin these studies ahead of time. Two rounds of affinity selection have been completed. This objective can be performed simultaneous to the Fab isolations.

Phage (approximately 10^{10}-10^{12} virions) from a phage display library displaying random 15-mer peptides on its pIII coat protein, were added to each dish of cells containing growth media. Affinity selection was performed similar to that for the Fab libraries outlined in Task 2, Objective 2.

Following the wash stages, specifically bound phage were eluted with resuspension and gentle agitation. A small portion of the eluted phage was used to produce serial dilutions of the phage which were subsequently used to infect K91 Blue Kan *E. coli* cells. Cells were then plated on agar plates containing kanamycin and tetracycline - K91 Blue Kan cells carry resistance to kanamycin and the phage that infect the cells will confer tetracycline resistance to those cells. A portion of the first round eluted phage was used to infect *E. coli* cells which were subsequently used to produce phage particles to be used as the input phage for biopanning round #2. We have finished two rounds of selection with MDA-MB 435 and MDA-MB-468 breast cancer cell lines.

Task 2: Months 29-30: Characterize binding using immunofluorescence and fluorescence titration.
To be done.

Task 3: Months 31-36: Chemically synthesize selected peptides and determine binding properties to breast cancer cell lines.
To be done.

(7) KEY RESEARCH ACCOMPLISHMENTS

* Affinity Selection of Random HCDR Fab and scFv libraries Against T Antigen.

* Micropanning of Six Fab clones.

* Determine DNA and Amino Acid Sequence of 5 Fab clones.

* Develop ELISA to Screen for T-binding.

* Purify anti-T scFv and Examine Binding by ELISA.

* Begin Screening Antibody and Peptide libraries for Molecules that Bind Lobular and Ductal Breast Cancer Cell Lines.

(8) REPORTABLE OUTCOMES

* Generate a Anti-Breast Cancer Serum and Splenic RNA Repository.

* DOD Breast Cancer Postdoctoral Fellowship Applied for by DOD-supported Student.

(9) CONCLUSIONS

Alternative detection, diagnostic, and therapeutic approaches are needed to help reduce the morbidity and mortality of breast cancer. Our approach is to employ combinatorial human Ig and peptide libraries to generate molecules specific to antigens present on breast cancer cells. The Fab and peptides will be valuable tools that will expedite basic and applied research into defining the determinants of breast cancer, that once radiolabeled, may facilitate early detection and diagnosis. Human IgG random CDR combinatorial Fab phage display libraries and more diverse scFV libraries were screened for Fab that bound the breast cancer-associated T antigen presented as a BSA-T antigen conjugant or asialofetuin. Numerous Fab and scFv-displaying phage were selected in this procedure and analyzed for binding to T antigen using ELISA. Of the approaches utilized, the scFv expressed the highest affinity and specificity for T antigen. The scFv may be superior to the Fab clones due to the increased diversity in the original scFv library versus the Fab libraries. Thus, future selection experiments with the scFv libraries may yield the best anti-T antibody fragments. Experiments are in progress to determine the epitope recognized by these selected antibody fragments. Two other approaches are being employed to obtain anti-T specific Ig. We are shuffling CDRs of a non-specific Fab in the hopes of converting it to a T-binding antibody. Second, we are isolating IgM from mice and hoping to humanize and convert to IgG molecules using molecular biological approaches. It is recommended that this aspect of the proposal be augmented. The untimely death of our collaborator Dr. George Springer, left us without the anti-T IgM murine monoclonal antibodies. Thus, we propose to generate our own using the University of Missouri Hybridoma Facility to assist us. Once generated, we would like to clone and express these antibodies using our well documented RT-PCR techniques (5). These problems can also be addressed through the simultaneous screening of peptide libraries for molecules that bind T antigen or breast cancer cells since peptides may be as or more valuable as diagnostic or therapeutic agents. We suggest that affinity selection of peptide libraries can begin earlier than originally planned since breast cancer cell lines that are currently in use in Fab library screening can also be employed in this approach.

"So What Section"

-Recombinant antibody fragments that bind T antigen may be useful reagents in breast cancer diagnosis, detection, or localization.
-Recombinant antibody fragments can be engineered to contain a radiometal chelation sequence such that radiolabeling with Re186/188 can yield radiopharmaceuticals that could destroy cancer cells.
-Recombinant antibody fragments that bind ductal or breast cancer cells may be used to identify new breast cancer antigens.
-Recombinant antibody fragments that bind ductal or breast cancer cells may be used to differentiate ductal from lobular breast cancer which can assist in developing appropriate therapeutic treatment regimens.

- Peptides that bind breast or ductal breast cells may be used to differentiate ductal from lobular breast cancer which can assist in developing appropriate therapeutic treatment regimens. Peptides may be tolerated in higher doses in patients than antibodies.

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